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## INTRODUCTION

Approximately two-thirds of breast cancers express the estrogen receptor (ER) and are stimulated by estrogens. For these tumors, the therapeutic strategies include estrogen ablation or antiestrogens. The molecular mechanism of hormone resistance in what are originally considered as hormone sensitive tumors is poorly understood. Tamoxifen is the most popular antiestrogen currently in use. The action of antiestrogens such as tamoxifen is a complex mixture of antagonism of the mitogenic action of estrogen at the level of ER in addition to a range of ER-dependent and independent actions. Changes in the ER-mediated gene expression may be associated with at least one form of estrogen resistance. DNA methylation of CpG islands has been a widely noted phenomenon in the mechanisms of gene silencing during the development of drug resistance.

Recent studies demonstrated that methylation of ER gene CpG islandmarks loss of ER expression in ER negative human breast cells, and treatment with hypomethylating agents such as 5-azacytidine reinstated the expression of ER (1, 2). ER+ phenotype not only requires expression of ER expression but also parallel changes in the transcription of several other genes regulated in an identical manner. We hypothesized that specific, adaptive, epigenetic changes in the methylation of CpG islands, are brought about during antiestrogen treatment in ER<sup>+</sup> breast tumor cells. Very limited amount of information is available on the genome-wide DNA methylation profiles. We proposed to employ a newly developed, powerful method called Restriction Landmark Genome Scanning (RLGS) for systematic detection of DNA methylation in breast tumor cells treated with antiestrogens.

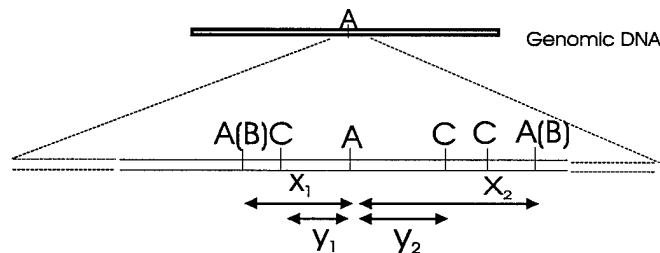
The experimental approach is to employ high resolution genome scanning by RLGS coupled with methylase-sensitive/insensitive restriction enzyme digestion of genomic DNA from the corresponding cell lines. Such analyses is expected to reveal DNA methylation at the genome level (3, 4, 5). Simultaneous probing with known target genes regulated by CpG methylation (ER) would serve to evaluate the relevance and specificity of genome-wide de/methylations observed by RLGS. The key to the success of this project is in obtaining reproducible patterns of restriction digested genomic DNA and high resolution, two-dimensional gel electrophoretic separation of the restriction fragments. Two additional features that this high sensitivity method relies on are: (a) the high specific activity labeling of Not-I landmarked DNA ends, and (b) complete/reproducible restriction enzyme digestion of agarose-bound DNA after the first dimension resolution, prior to electrophoresis in the second dimension. These methods are relatively new and scant details of working procedures have been described (3, 4, 5). Some of the recent studies utilized the power of the RLGS technique to reveal genome wide DNA alterations including methylations (6-9).

We have been successfully utilizing the Iso-Dalt equipment from the Large Scale Biology for high resolution analysis of two-dimensional protein profiles (2D-gel core facility at the Lombardi Cancer Center). In our pilot studies, submitted with the proposal application, we showed preliminary promise of being able to adapt the Iso-Dalt equipment to resolve restriction landmarked genomic DNA in first dimension, digest the agarose-bound DNA with methylase-sensitive/insensitive restriction enzymes followed by electrophoresis in the second dimension. In the present report, we describe our efforts at optimizing the conditions for the performance of RLGS.

## Brief description of the RLGS method according to the present approach

High molecular weight genomic DNA from MDA-MB-231 cells (Estrogen receptor negative) was digested in solution with Not I (Enzyme A) and a second rare cutting enzyme of choice such as EcoRV (Enzyme B). Subsequently, the DNA was labeled at the Not I ends by a sequenase reaction incorporating  $\alpha$ -<sup>32</sup>P- dGTP and  $\alpha$ -<sup>32</sup>P- dCTP. This reaction is the key to the entire procedure of genome scanning, following the labeled Not I ends as landmarks distinct from the other restriction enzyme cleavage sites introduced later on in the DNA. Such landmarked, sequenase-labeled DNA was separated on disc gels using the Iso-Dalt apparatus. The gel was equilibrated with appropriate buffer and incubated with a third restriction (Enzyme C) enzyme to carry out in-gel digestion of DNA prior to resolving in the second dimension. Thus, size separation in two dimensions, combined with digestion with three restriction enzymes of genomic DNAs of different origin or of the same origin subjected to DNA modifications are expected to result in unique DNA restriction patterns.

Shows a scheme of the procedures  
taken from Electrophoresis 1995, 16, 197-202.



A: Site for restriction enzyme A (Restriction landmark)

B: Site for restriction enzyme B

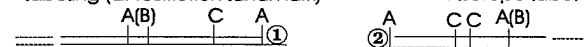
C: Site for restriction enzyme C

↓ blocking (with analogues of nucleotide)

↓ landmark cleavage (with restriction enzyme A)

↓ labeling (at restriction landmark)

①②: Isotope label



↓ the fragmentation (with restriction enzyme B)

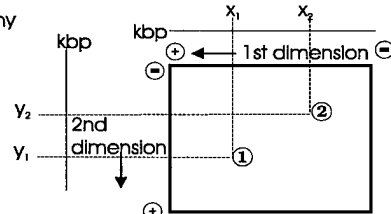
↓ the first fractionation (by 0.8-1% agarose gel electrophoresis)

↓ the fragmentation of labeled DNA with restriction enzyme C

↓ the second fragmentation

(by 5-6% polyacrylamide gel electrophoresis)

↓ autoradiography



Procedure of the RLGS method

## EXPERIMENTAL PROCEDURES

### Restriction Landmark Genome Scanning using Iso-Dalt equipment

We describe the basic procedure adapted in our laboratory and comment on the technical difficulties experienced during the procedures.

#### Creating restriction landmarks in genomic DNA:

1. Blocking of free ends of DNA: High molecular weight genomic DNA was blocked (5-10  $\mu$ g) in a reaction containing 0.4  $\mu$ M dGTP[ $\alpha$ ]S, 0.2  $\mu$ M dCTP[ $\alpha$ ]S, 4.0  $\mu$ M ddATP, and 4.0  $\mu$ MddTTP. The reaction buffer is made up of 50 mM tris-Hcl, pH 7.4, 10 mM MgCl<sub>2</sub>, 0.1 M NaCl, and 1 mM DTT in the presence of 1.75 units of DNA polymerase I at 37 °C for 30 min. The reaction was terminated by boiling to 65 °C for 30 min. The DNA was ethanol precipitated in preparation for the landmark digestion.
2. 5.0-10.0  $\mu$ g of blocked DNA was digested with restriction enzymes Not I ( 50 units) and EcoRV (120 units) (Enzymes A and B) at the same time in an overnight reaction, at 37 °C in 150  $\mu$ l reaction mixture (both of these enzymes are maximally active in the same buffer). It is only the Not I ends that incorporate the  $\alpha$ -<sup>32</sup>P- dGTP and dCTP. EcoRV produces blunt ends that can not be labeled by the ensuing sequenase reaction.
3. 2  $\mu$ l of sample digest was analyzed on an agarose mini gel. If digestion is not complete, more enzyme was added and digestion further continued. Overnight incubation usually resulted in complete digestion.
4. The digested DNA was subjected to ethanol precipitation by addition of 1/10 th volume of 3.0 M sodium acetate, and 2.5 volumes of absolute ethanol at -20°C for 2-4 h followed by two quick washes of 70% ethanol (1 ml each). The DNA pellet was dissolved in H<sub>2</sub>O (0.5-1  $\mu$ g DNA/ $\mu$ l) thoroughly by vortexing or pipetting.
5. Sequenase labeling reaction of the Not I ends of DNA was carried out for 30 min at 37 °C with 50  $\mu$ Ci of  $\alpha$ -<sup>32</sup>P-dCTP ( 3000 Ci/mmol) and 50  $\mu$ Ci of  $\alpha$  - <sup>32</sup>P-dGTP (3000 Ci/mmol), in presence of 8 units of sequenase. Once again, the labeled DNA was ethanol precipitated followed by two successive 70% alcohol washes in order to remove unincorporated nucleotides. At this point DNA was dissolved in 20  $\mu$ l TE buffer for subsequent electrophoresis.

#### First Dimension Electrophoresis

1. Iso-Dalt equipment was used for the first dimension gel electrophoresis to perform disc gel resolution of landmarked and sequenase labeled DNA. The glass tubes for use in the first dimension DNA resolution were coated with 1.0 % BSA solution and allowed to dry. The gel tubes were arranged in the upper buffer chamber of the Iso-Dalt apparatus, and cut edges of

(0.5 cm long) pipet tips (200  $\mu$ l) were inserted at the lower end of the glass tubes. Without this plug, agarose gel tubes slip down when allowed to stand in vertical position. A gel solution of 0.8% agarose (Seakem agarose-FMC) was prepared in 1 X TAE by boiling for 3 min in a microwave oven. The final volume was readjusted and cooled to 50  $^{\circ}$ C before pouring. 1.0 ml syringes fitted with appropriate tygon tubing (2-3 inches each ) were afixed on top of the gel tubes and the agarose solution, held at 50  $^{\circ}$ C, was pulled to rise up to top of the gel tube leaving 3-4 cm on the top of the tube empty for sample loading. When the agarose tubes are set, the apparatus was filled with 3.6 liters of 1 X TAE.

2. 1-2  $\mu$ g of digested/ sequenase labeled DNA in 24  $\mu$ l of TE was mixed with 6  $\mu$ l of 5 X loading buffer. Throughout all the procedures heat denaturation of DNA is not allowed because all the restriction digestions are dependent on the sequence of intact double stranded DNA. 10  $\mu$ l aliquots of this DNA were loaded into each of the tubes while loading 1 Kb extension ladder (markers from BRL- to cover an extended range of 1-40 Kb) and Hindi III cut lambda markers in other tubes. The electrophoretic run was commenced at room temperature at 35 V. Usually for a 16 cm gel it takes 20 h or 30 h for a 24 cm gel.
3. Upon completion of the run, the gel tubes were removed, the marker tubes were stained with ethidium bromide. It is important to mark the ends of the sequenase- labeled DNA tubes when expelled from glass tubes.

### **In gel digestion of landmarked DNA with restriction enzyme C**

1. The gel tubes were exposed to X-ray film to make certain that the landmarked DNA is extremely hot and resolved in the expected molecular size range. The agarose gel tubes were equilibrated (in parafilm boats) against several changes of the appropriate restriction enzyme buffer for a total of 3 h . Subsequently, the gel tubes were transferred to petri dishes with 1.0-1.5 ml of fresh restriction enzyme buffer containing 100  $\mu$ g/ml BSA and placed on ice bath for 15 min. Between 250-300 U of enzyme C (Pst I) were added while mixing the contents by rocking.
2. The petri dishes containing tubes were slowly rocked overnight at 5  $^{\circ}$  C such that the fluid phase moves back and forth along the edges of the gel strip. Later on, the gel tubes were moved to a stationary 37  $^{\circ}$ C water bath for 3-4 h. At this time addition of a batch of fresh enzyme is highly recommended. At the end of this digestion, the enzyme solution was drained off and the gel tubes were floated into 50 ml of 1 x TAE.

### **Second dimension gel electrophoresis:**

We decided to use agarose or acrylamide for the second dimension as we learn to expect the size of the fragments generated by the enzyme C.



1. The second dimension gel was poured with BRL agarose (1.2%) in 1 X TBE as a bed of 20 x 20 cm in a horizontal gel apparatus to a depth of 0.4 cm. A trough of 0.2-0.3 cm width was formed at the top of the gel by placement of a transverse plastic bar when gel is poured. The trough should be designed to fit the gel tube from the first dimension. The second dimension gel was also made up of 5 % acrylamide on certain occasions in 1X TBE. Agarose tubes after the first dimension electrophoresis were transferred onto the slab and electrophoresis was performed (vertically).
2. Transferring the gel tube to the second dimension gel requires particular care. The buffer from the gel tube was drained by careful decanting or aspiration, leaving just enough fluid to slide the gel tube easily. Against a dark background, the gel tube was maneuvered onto a plastic sheet long enough to fit into the gel trough, and positioned along the edge of the gel trough (identifying the origin and the end of first dimension run). There is no sidedness to the gel tube, however one needs to make certain that the gel tube adheres to the forward gel surface of the trough. 0.5-1.0 ml of 0.5% agarose in 0.5 X TBE was pipetted into the open space between the gel strip and the front of the trough. The gel tube is gently advanced to the front edge of the trough where it should remain attached as the gel sets. Care was taken to avoid trapping bubbles between the gel tube and the running gel since this will interfere with the DNA mobility. The plastic strip was withdrawn, and 1-2  $\mu$ l of molecular weight marker was loaded in the form of liquid in a well created for the purpose or embedded in agarose directly into the ends of the embedded gel tube.
3. The second dimension gel (20 x 20 cm) was subject to electrophoresis at 40 V for nearly 20 h. After the electrophoresis, the gel was transblotted to nylon membrane in 1 X TBE using an IDEA scientific transblot Genie apparatus for 2 h transfer at 7 V/350 mamps. The membrane was autoradiographed at -70 °C. The use of this transblot apparatus in case of agarose gels used for the second dimension was a big improvement in being able to transfer the profile of restriction digestion patterns to a membrane easy for autoradiography and storage for further use.

## RESULTS

We provide our results in the form of figures 2, 3, and 4 while explaining the outcomes of our attempts at adapting the Is-Daft equipment to perform RLGS. These three figures bring out the salient points of our progress as:

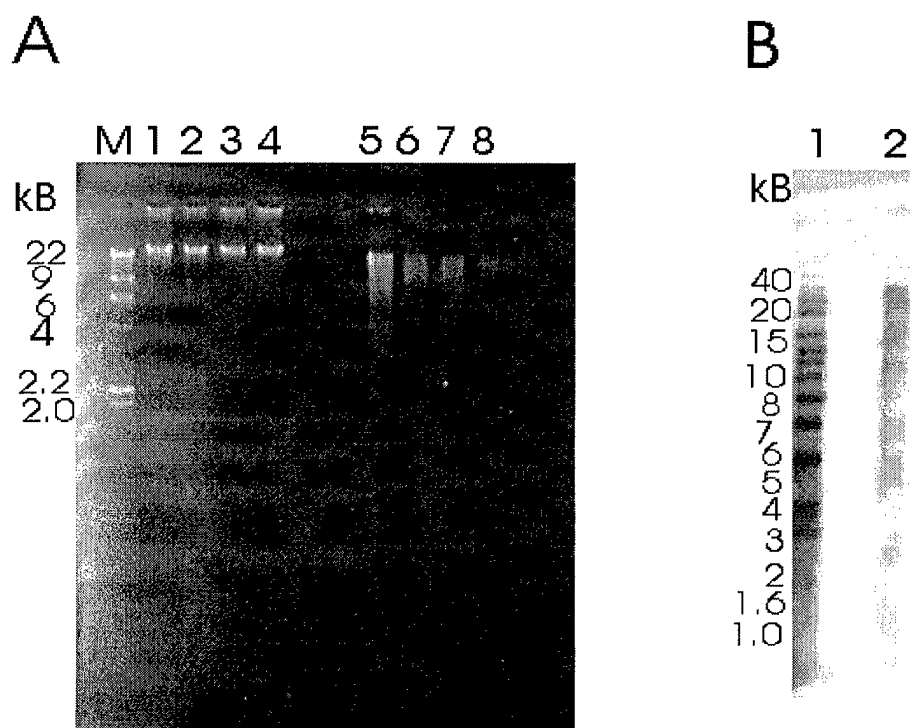
1. Complete landmark digestion of genomic DNA with Not I and EcoRV (enzymes A and B);
2. Sequenase labeling of landmarked Not I ends of genomic DNA with radioactive dCTP and dGTP;
3. Digestion of gel-bound DNA with restriction enzyme C (1 Kb DNA markers);
4. Resolution of 1-40 Kb DNA on disc gels (genomic as well as 1 Kb markers) using Is-Daft apparatus;
5. Second dimension resolution using both 5% acrylamide as well as agarose gels;

6. Transfer the two-dimensional DNA resolution patterns to nylon membranes using 2 h Southern transfers;
7. Efforts to achieve complete digestion of gel-bound sequenase labeled genomic DNA are not yet satisfactory.

Each and every one of these steps required special attention unlike conventional one dimensional DNA gel separations. Particularly, Southern transfers in 2 h was a much needed improvement in order to perform several gels and obtain 2D-profiles in a time frame (within the 14 day half life of  $^{32}\text{P}$ -nucleotides) feasible to make comparisons of MDA-MB-431 cells treated with various agents.

We circumvented certain difficulties in the following ways:

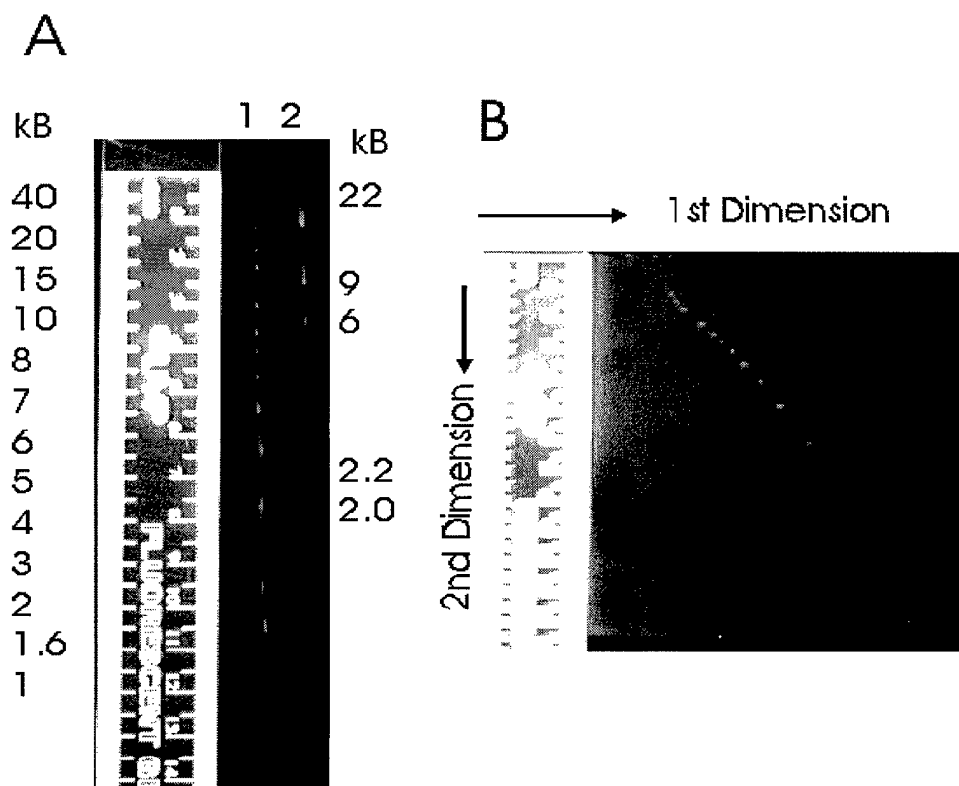
1. To drive the in-gel restriction digestion reaction to completion, we added the >2000 units of enzyme per gel tube, in 2-3 batches carrying out the digestion overnight.
2. Decision to use 5% acrylamide- or 1.2% agarose- gels for the second dimension was made on a case by case basis. We switched to 1.2 % agarose gels instead of the 5% polyacrylamide gels when we learned that our fragments from the digestions were larger than 1.0 Kb.
3. Our attempts to adapt composite gels (agarose-acrylamide) for second dimension resolution (to achieve resolution of both high and low molecular weight DNAs) were not successful. We concluded that these acrylamide-agarose composite gels provided only marginal improvement in the resolution of > 1 Kb fragments while they were not amenable to drying or transblotting for subsequent autoradiography.
4. The RLGS procedures involve an entire week, while autoradiography requires an additional week. Isotopes such as  $\alpha$ - $^{32}\text{P}$ -CTP and  $\alpha$ - $^{32}\text{P}$ -GTP with short half life were extremely inconvenient for the lengthy experimental procedures and hence we have planned to use nonradioactive detection methods and use of  $^{35}\text{S}$ -labeled nucleotides in future.



**Figure 2:** Panel A shows digestion of genomic DNAs from MDA-MB-431 cells with Not I and EcoRV. Lanes 1,2,3,and 4 represent high molecular weight DNAs from control MDA-MB-231 cells and those treated with estradiol, tamoxifen and 5-azacytidine respectively. Lanes 5, 6, 7 and 8 demonstrate the extent of digestion in each case. The digestion reaction consisted of 5  $\mu$ g of blocked DNA, in 1x NE buffer 3 in presence of 50 units of Not I and 100 units of EcoRV at 37  $^{\circ}$ C overnight. NE buffer 3 (100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM  $MgCl_2$  is the appropriate buffer for both of the enzymes and hence DNA was digested with them simultaneously. 0.5  $\mu$ g DNA samples were subject to electrophoresis on 0.8 % agarose gels and stained with ethidium bromide. Lane marked M indicates lambda markers cut with Hindi III.

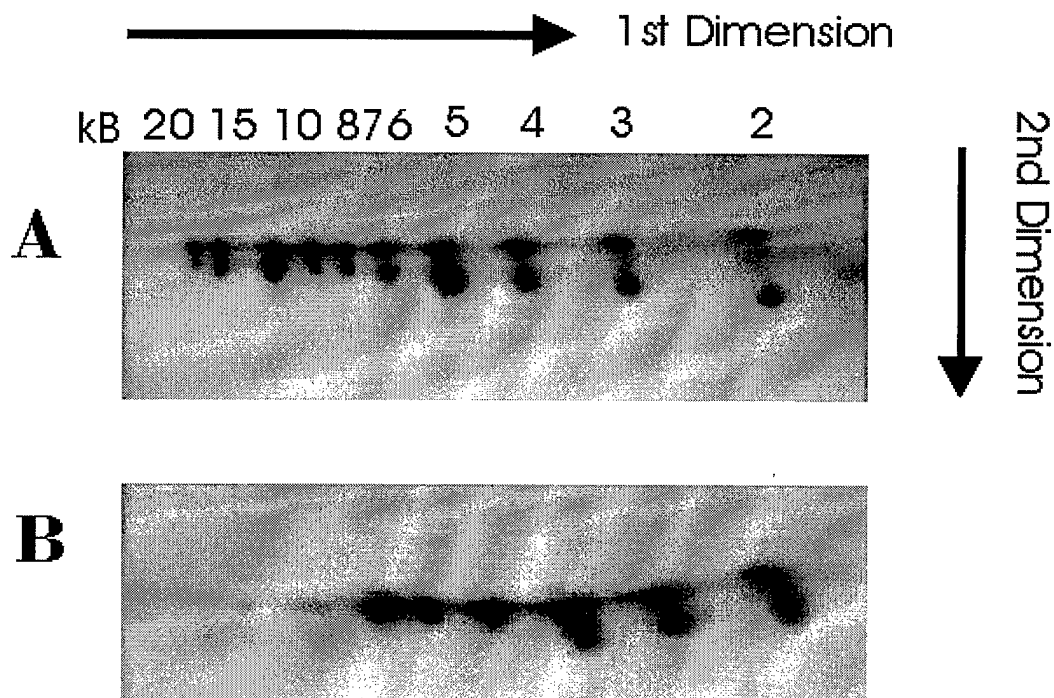
**Panel B.** 1 Kb extension ladder (consisting of DNA markers ranging 1-40 Kb) was random prime labeled (with Klenow fragment) with 25  $\mu$ Ci of  $^{32}$ P-dCTP and resolved on 0.8 % seakem agarose gel (lane 1). These sizes of the markers were used to represent approximate sizes of genomic DNA fragments resulting from Not I and EcoRV digestions. Lane 2 is landmark digested DNA labeled with sequenase in presence of 50  $\mu$ Ci of  $^{32}$ P-dCTP and 50  $\mu$ Ci  $^{32}$ P-dGTP. The DNA from the agarose gel was transferred on to nylon membrane in a 2 h procedure of Southern transfer in 1 X TBE in an IDEA scientific genie blotter prior to autoradiography.

While attempting to adapt the Iso-Dalt apparatus to resolve high molecular weight DNA in disc gels we used the Is- Daft apparatus and 1 Kb extension ladder to determine the utility of the equipment for the intended use of restriction landmark genome scanning.



**Figure 3:**

Figure 3A, shows resolution of 1 Kb extension ladder (lane 1) and lambda markers (lane 2) on 0.8 % seakem agarose tubes resolved for 20 h at 30 V in 1.5 mm internal diameter/20 cm long agarose tube gels cast in gel tubes suited for isoelectric focussing of proteins. Electrophoresis was carried out at room temperature for 20 h in 3.6 liters of 1 X TAE. The tubes were extruded and stained with ethidium bromide. The markers resolved in the form of a diagonal in Panel B represent second dimension resolution of these 1 Kb markers on an 0.8 % agarose gel and ethidium bromide stained.



**Figure 4:** 1 Kb extension ladder markers were random prime labeled (Klenow fragment) with  $^{32}\text{P}$ -dCTP. They were resolved on tube agarose (0.8%) gels, digested *in situ* with EcoRI (800 units) (Panel A) or Pst I (800 units) (Panel B) and subjected to second dimension resolution on 5% acrylamide gels.

Note: Note the differences in the restriction patterns with the two enzymes EcoRI and Pst I respectively of the various marker DNA fragments.

## CONCLUSIONS

In order to establish the methods for reproducible performance of RLGS, we adapted the Is-Daft equipment. Although the procedure is simple in concept, involves many manipulations, thus the chances of failure are high in the beginning. Close attention to detail and a cautious attitude toward sudden failure are useful in persevering to a good series of high resolution gels. Further studies are in progress to compare and contrast the DNA methylation profiles of ER<sup>+</sup> and ER<sup>-</sup> breast tumor cells treated with tamoxifen.

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